IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application Of:	)	
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Roger S. CUBICCIOTTI	)	Group Art Unit: 1645
	)	
Application Number: 10/020,151	)	Examiner: Jeffrey R. SNAY
	)	
Filed: December 18, 2001	)	Confirmation No. 7118
	)	
For: MODIFIED PHYCOBILISOMES AND USES THEREFORE	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF MR. JOHN MORSEMAN UNDER 37 C.F.R. § 1.132

I, Mr. John Morseman, declare:

1. THAT, I am the Director and Product Manager of Fluorescent Products at Martek Biosciences Corp., located in Columbia, Maryland. Martek Biosciences Corp. is an exclusive licensee of the subject invention.

2. THAT, I received an M.S. in environmental science from Johns Hopkins University and a B.S. in biology from Loyola College of Maryland. I have studied algae and algal pigments for over ten years. I am an author of six peer-reviewed articles relating to phycobilisomes and phycobiliproteins.

3. THAT, I am familiar with the contents of the documents in Exhibits 1 and 2 as well as the above-identified application (Serial No. 10/020,151) (the "Application"). References to the Application's Specification are made with reference to the Application as published in U.S. Pub. No. 2003/134325.

4. THAT, I am familiar with the experiments described in the Examples described in the Application, including Examples 2-4 described in paragraphs [0069] through [0086], and I have replicated many of these experiments.

5. THAT, U.S. Patent No. 4,857,474 to Waterbury et al. entitled "Phycoerythrins useful in fluorescent conjugates" (the "Waterbury patent") and attached hereto as Exhibit 1, describes phycoerythrins, a component of phycobilisomes. The Waterbury patent also mentions isolated phycobilisomes "prepared as described by Yamanaka et al. (J. Biol. Chem. 253: 8303 (1978)) ("Yamanaka article") with the exception that the breakage buffer contained 0.01M EDTA and 0.001 M phenylmethylsulfonyl fluoride." Waterbury patent, column 7, lines 17-21. The Yamanaka article is attached hereto as Exhibit 2.

6. THAT, the Yamanaka article describes a "procedure...for the preparation of stable phycobilisomes from the unicellular cyanobacterium *Synechococcus* sp. 6301 (also known as *Anacystis nidulans*)." Yamanaka article, page 1.

7. THAT, the Yamanaka article shows that the energy transfer properties of phycobilisomes depend on the preservation of the phycobilisomes' structure as a cluster of rods. As stated in the Abstract of the Yamanaka article:

> Electron microscopy shows that the phycobilisomes are clusters of rods. The rods are made up of stacks of discs which exhibit the dimensions of short stacks made up primarily of phycocyanin [citation omitted]. Loss of the clusters, by dissociation into rods under suitable conditions, is associated with loss of energy transfer as shown by a shift in fluorescence emission maximum to 652 nm.

Further, the Yamanaka article notes that the "preservation of the rod clusters is essential for retention of energy transfer [to allophycocyanin]." Yamanaka article, p. 8308. For a sample of phycobilisome material prepared according to the Yamanaka article:

> Electron microscopy of the sample showed that even [a] brief exposure to a buffer at low ionic strength and a pH of 5.5 caused dissociation of the clusters into rods and discs (compare Fig. 5, B and C). Although prolonged residence, 6 h, of the phycobilisomes in

> this buffer caused the complete dissociation of rods into discs (Fig. 5D), no further change occurred in the fluorescence emission spectra. Thus, we conclude that the preservation of the clusters is essential for retention of energy transfer through allophycocyanin and allophycocyanin B.

Yamanaka article, p. 8308.

8. THAT, the phycobilisomes investigated in the Yamanaka article dissociated into their component parts and therefore did not preserve the phycobilisomes' rod clusters. This dissociation is documented at least in Figures 5A-5D of the Yamanaka article, which illustrate electron micrographs of preparations of phycobilisomes. Figure 5A shows approximately intact phycobilisomes diluted to 40 μg/ml in 0.75 M Na-K-PO_4, pH 8.0, negatively stained. Figure 5B shows phycobilisome material as prepared in Figure A, but contrasted by rotary shadowing. Figure 5C shows phycobilisome material prepared as in Figure 5B, but 15 minutes after dilution into 0.1 M sodium acetate, pH 5.5, wherein clusters have mostly disappeared. Figure 5C shows that only single discs and rod-like stacks of discs are remaining. Figure 5D shows phycobilisome material 6 hours after dilution into 0.1 M sodium acetate, pH 5.5, negatively stained. In Figure 5D, single discs predominate, along with some short stacks of discs. Accordingly, the phycobilisomes of the Yamanaka article, at least as shown in Figures 5A-5D, did not remain intact or stabilized. As further noted in the Yamanaka article, "30 s after extensive dilution into 0.02 M Na-K-PO_4, pH 8.0, a phycobilisome preparation showed no clusters." Yamanaka article, p. 8307.

9. THAT, the dissociation of the phycobilisomes prepared in the Yamanaka article was caused at least in part by a decrease in ionic strength, and it resulted in a loss of the phycobilisomes' energy transfer properties. As shown in Figure 5C, a decrease in ionic strength (by dilution of the phycobilisome preparation into 0.1 M sodium acetate) disrupts intra-phycobilisome energy transfer, as indicated by the fluorescence emission maximum's "shift from

673 to 652 nm, indicating virtual absence of energy transfer to allophycocyanin and allophycocyanin B (see Fig. 6, *inset B*)." Yamanaka article, p. 8307-8308. The Yamanaka article additionally notes that "the fluorescence emission properties of phycobilisomes are drastically affected by dilution into 0.1 M sodium acetate, pH 5.5...." Yamanaka article, p. 8307.

10. THAT, the Yamanaka article identifies the conditions for maintaining stability of unmodified phycobilisomes to be pH = 7.5-9.0 and high ionic strength. In particular, the Yamanaka article identifies the conditions of optimal stability for *Synechococcus* sp. 6301 phycobilisomes to be "pH 7.5 to 9.0, and a very high ionic strength," and this conclusion is "consistent with a number of observations made by other investigators on the physiology of this organism." Yamanaka article, p. 8309. The Yamanaka article states that a procedure employed by Gantt and Lipschultz resulted in "relatively stable phycobilisomes." Yamanaka article, p. 8309. According to this procedure:

> [Phycobilisomes are] prepared in 0.75 M Sorensen's phosphate buffer at pH 7.0 containing 1% Triton X-100. Under these conditions, the bulk of the phycobiliproteins migrate as a single rapidly sedimenting band on sucrose step gradients.
>
> [R]elatively stable phycobilisomes...could by obtained by the [Gantt and Lipschultz method]. The stability of [specific] phycobilisomes was then studied as a function of ionic strength, type of salt used, and pH. These exploratory experiments demonstrated that [specific] phycobilisomes showed optimum long term stability, as determined from the preservation of energy transfer, between pH 7.5 and 9.0, at ionic strengths equivalent to or higher than that of 0.75 M Na-K-PO_4, pH 8.0. These phycobilisomes were much less stable at pH 7.0.... This procedure leads to little if any dissociation of phycobilisomes and results in particles with a fluorescence emission maximum at 673 nm.

Id.

11. THAT, consistent with the Yamanaka article's finding that phycobilisome stability required a high ionic strength environment, the Specification reports that phycobilisomes were disrupted by a decrease in ionic strength. This disruption of the

phycobilisomes was detected by disruption of intra-phycobilisome energy transfer. As stated in the Specification:

> In agreement with published studies (e.g., Katoh (1988) Phycobilisome stability, in Methods in Enzymology Vol. 167, pp. 313-318, Academic Press; and Gantt et al., 1979, supra), isolated phycobilisomes were shown to be unstable to decreases in protein concentration and ionic strength. Using *P. cruentum* phycobilisomes, for example, intra-phycobilisome energy transfer was disrupted within minutes following dilution of protein (below about 1 mg/ml) or buffer (below about 0.5 M KPi), as exhibited by concentration-dependent decreases in the ratio of 666/573 nm fluorescence emission with 545 nm excitation. Similar dissociation was observed for phycobilisomes isolated from *Spirulina platensis* and *Anabaena variabilis* based on a decrease in emission of the terminal acceptor. To enable reproducible preparation of stable phycobilisome-labeled ligands and receptors for use in conventional specific binding assay configurations, phycobilisomes are preferably stabilized against dissociation.

Specification, paragraph [0060].

12. THAT, under most conditions expected for use of phycobilisomes, and in most uses disclosed in Applicant's specification, the high ionic strength environment necessary to prevent dissociation of the phycobilisomes will not be maintained.

13. THAT, the Applicant's Specification discloses "stabilized phycobilisomes" that maintain stability in the absence of high ionic strength solutions. The Application defines "stabilized phycobilisomes" as follows:

> "Stabilized phycobilisomes" are stable even under conditions of dilute ionic strength (<0.5 M) and protein concentration (<1 mg/ml), in contrast with native phycobilisomes. In addition, they are stable in the presence of glycerol, sucrose, and polyethylene glycol. Typically, the phycobilisomes are stabilized by means of a gentle crosslinking treatment, such as with formaldehyde or very low concentrations of glutaraldehyde. Other medium-, short- or zero-length crosslinking reagents may also be used.

Specification, paragraph [0028].

14. THAT, the "stabilized phycobilisomes" disclosed in Applicant's Specification may be prepared according to at least the following methods disclosed in Applicant's Specification:

> Stabilization methods which are embraced by the present invention include covalent as well as non-covalent means. Covalent methods include crosslinking and multi-point attachment of polymers that span at least two phycobilisome constituent proteins. Crosslinking agents may be zero-length (involving the direct attachment of two phycobilisome groups without intervening spacer atoms) or they may include spacer arms of varying length. Non-covalent stabilization may be accomplished using cosolvents, such as salts and sugars, hydrophobic or affinity-based interactions, such as with certain polymers or polyvalent receptors, entrapment or encapsulation (e.g., using gels, liposomes, or micelles), or changes in physical state, such as freezing or dehydrating. Suitable methods for stabilizing phycobilisomes include the methods discussed below.

Specification, paragraph [0061]. Phycobilisomes prepared according to these methods are "stabilized phycobilisomes" as defined in the Specification at least in part because they are "stable even under conditions of dilute ionic strength (<0.5 M) and protein concentration (<1 mg/ml)". Specification, paragraph [0028]. These above-described preparation methods such as "crosslinking" and "multi-point attachment of polymers" are not disclosed in the Yamanaka article.

15. THAT, Applicant's Specification reports that phycobilisomes stabilized according to Applicant's Specification retain intra-phycobilisome energy transfer upon (1) decrease of ionic strength (as shown in Examples 2 and 3 of the Specification) or (2) drying (as shown in Example 4 of the Specification). Specification, paragraphs [0069] through [0086]. The retention of the phycobilisomes' desirable intra-phycobilisome energy transfer properties were verified by measurements of the relative intensity of the fluorescence emission peaks at 666 nm and 573 nm. Id.

16. THAT, the phycobilisomes disclosed in the Yamanaka article were not "stabilized phycobilisomes" as defined in the Specification at least because Yamanaka's "stable" phycobilisomes were maintained at "high ionic strength" (such as ionic strengths "equivalent to or higher than that of 0.75 M Na-K-PO_4"). Yamanaka article, p. 8309. The "stable" phycobilisomes disclosed in Yamanaka are <u>not</u> "stable even under conditions of dilute ionic

strength (<0.5 M) and protein concentration (<1 mg/ml)" as required by Applicant's definition of "stabilized phycobilisomes." Specification, paragraph [0028]. As mentioned above, the phycobilisomes disclosed in the Yamanaka article remained stable only under conditions of high ionic strength, and therefore the phycobilisomes disclosed in the Yamanaka article were not able to maintain the phycobilisomes' desirable light-transmitting properties in conditions of low ionic strength. Therefore, Yamanaka does not disclose "stabilized phycobilisomes" as defined in the Specification.

17. THAT, the dissociation and instability problems identified with respect to the phycobilisomes in the Yamanaka article apply equally to the phycobilisomes prepared according to the Waterbury patent. The fact that the phycobilisomes of the Waterbury patent were prepared as described by the Yamanaka article "with the exception that the breakage buffer contained 0.01M EDTA and 0.001 M phenylmethylsulfonyl fluoride" does not change this conclusion. Waterbury patent, column 7, lines 17-21. The use of a different breakage buffer does not change the phycobilisomes of the Waterbury patent in any significant way with respect to instability and dissociation.

18. THAT, the data described above demonstrate that none of the preparations according to the Yamanaka article and the Waterbury patent possess the characteristic properties of phycobilisomes stabilized according to Applicant's Specification. No preparation of phycobilisomes made by the method of Yamanaka or Waterbury displays both the required stabilization of phycobilisome fluorescence and the required homogeneity or solubility.

19. THAT, all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 11/17/05

Mr. John Morseman